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An Agent that can Prohibit Microbial Development and Infection

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Abstract

We have developed a process that makes use of double-stranded DNA:RNA hybrids to inhibit specific, targeted genetic activity completely within a cell. This process can be used in both human and bacterial cells. The agent that produces this effect can be inserted into a cell and remain quiescent for a considerable period of time, without affecting cellular processes, until the gene against which it is targeted is induced. At this time the agent becomes effective, silencing the genetic response without affecting the host cell in any other way. When given as an anti-infective, this process may have significant use as an anti-bacterial, anti-viral agent. Our objective with this proposal is to develop the siHybrid concept sufficiently that it can be used as both an antibiotic and an antiviral agent.

Introduction/Background

The phenomenon of short interfering RNA (siRNA) molecules has provided a powerful tool for exploring gene function. However, it has been reported to be nonfunctional in bacteria [1]. The discovery of an equivalent mechanism in bacteria would provide a powerful tool for studying gene function, and potentially yield a new way to combat bacterial infectious diseases. Here we show that a new method using modified siRNA molecules promotes an attenuation of gene activity in bacteria similar to that observed in eukaryotes. The *Escherichia coli folA* and *lacZ* genes were targeted, as well as the Tn*9 cat* gene. Significant reductions in gene activity were observed in all three cases. The modified molecules can be added to growing or quiescent cells without transformation, making them simple and effective to use. The advent of gene silencing technology in prokaryotes represents an important new research tool and may reveal heretofore unknown biological mechanisms.

In recent years it has been accepted that RNA interference is mediated by siRNA, which exhibits sequence-specific gene silencing. Although the detailed mechanism of siRNA gene silencing is not fully understood, it is known that introduction of siRNA molecules into a cell causes degradation of mRNA to which the siRNA molecule shares sequence identity [2, 3]. siRNA has been shown to work most efficiently when the double-stranded RNA is 19-21 base pairs with 2-3 base 3' overhangs at each end [4]. siRNA-mediated gene silencing holds promise in many research and therapeutic arenas and is a marked improvement on antisense therapy.

Among the shortfalls of siRNA are the short duration of its effects, the difficulty of getting the molecules into cells and the fact that siRNA does not function in bacteria. The short duration stands to limit its use clinically; such as for cancer therapies, antiviral agents, and treatments for certain genetic diseases. Continuous production of siRNA by viral gene delivery systems may provide a means to sustain the effect. The lack of an effect on bacteria means that siRNA cannot be used as an antibacterial agent.

SiRNA molecules do not induce RNA interference-like responses in bacteria. This is believed to be due to the presence of RNase III in the cells [1]. *E. coli* RNase III is a double-stranded RNA-specific endonuclease encoded by the *rnc* gene that readily degrades dsRNA into an acid-soluble form. It has been shown to be involved in rRNA maturation and mRNA decay [reviewed in 5]. The RNase III-like nuclease, Dicer, has been implicated in the mechanism of mammalian RNA interference by cutting long, dsRNA into short ~22 nt RNA [6]. RNase H is known to digest the RNA strand of RNA:DNA hybrid sequences and is involved in the degradation of RNA primer sequences during DNA synthesis in mammalian cells and in prevention of undesired DNA synthesis from any non-*ori-C* primer sites in *E. coli* [reviewed in 7, 8]. Mammalian RNase H was shown to degrade hybrids consisting of antisense DNA and mRNA in the rabbit reticulocyte lysates system [9] and is believed to be one of the main factors in antisense-based gene silencing [reviewed in 10].

We have recently reported that changing the molecular composition of siRNA molecules from double-stranded RNA to an *RNA:DNA* hybrid (siHybrid) dramatically improves the degree and duration of the gene-silencing effect in eukaryotic cells [11]. Effects that lasted for two days with double-stranded RNA lasted for over a week with the hybrid molecule. We hypothesized that this is due to the higher stability of *RNA:DNA* hybrids in a cell.

Results/Technical Outcome

The significantly enhanced effects of these molecules relative to siRNA in eukaryotes suggested that it was worth testing their effect in bacteria. Our first target in bacteria was the Tn*9 cat* (chloramphenicol acetyl transferase) gene, which confers resistance to the antibiotic chloramphenicol and for these experiments was present on a common cloning vector. We performed a simple experiment in which increased doses of a RNA:DNA siHybrid were added to E. coli cultures. **Figure 1A** shows that with increased siHybrid concentrations, bacterial survival in the presence of chloramphenicol decreased dramatically, while siRNA of the same sequence had little effect. The control, non-homologous siHybrid and siRNA tests all exhibited statistically similar growth in the range of 32.0 - 45.0 x 10^7 CFUs. The results indicate that colony growth is inversely proportional to *cat* siHybrid concentration. The *cat* siHybrid concentrations at 1.0 μg/ml, 2.0 μg/ml and 4.0 μg/ml all showed around a 60 % decrease in colony formation compared to the control. The lack of effect of the siHybrid directed to mammalian glucose 6-phosphate dehydrogenase (G6PD) suggests that this is a gene-specific phenomenon. Relative values of *cat*

protein present in each sample were obtained by representing the average value of the positive control conditions as 100% and dividing the averages for the experimental conditions by the average positive control value. There was approximately a 40% reduction in the level of *CAT* protein in cells treated with 4 ug/ml *cat* siHybrid (**Figure 1B**). The discrepancy in results between the colony-forming assay and the CAT assay, detecting protein levels, is readily explained by the slightly different conditions under which the two types of experiments are carried out. Protein assays are done by immediately arresting and lysing the entire cell population, while colony-forming assays require a longer processing time, and a less robust assay. Satellite colony formation, and an unknown absolute duration of the siHybrid effects in bacteria, contribute to the difference.

To demonstrate further the specificity of this effect, we targeted the *folA* gene of *E. coli*, the product of which is essential in minimal medium where the cells must synthesize purines and pyrimidines *de novo*, and unnecessary in rich medium, where these compounds are in abundance. Inhibiting the action of folA is non-lethal; it merely prevents cells in minimal medium from undergoing DNA synthesis. When we added the *folA* siHybrid molecules to bacteria growing in rich medium there was no effect. In minimal medium, however, the siHybrid has a marked effect on growth, similar to the effect of trimethoprim, a widely used antibiotic that is a DHFR inhibitor. The similarity to trimethoprim hints at the possible use of this technology for antibacterial therapy. This effect was entirely eliminated when nucleosides were added to the medium, thus clearly demonstrating the specificity of the effect. To assess the relative numbers of cells affected by treatment, we added the *folA* siHybrid to the medium, and then added ampicillin. All cells that had acquired the siHybrid molecules stopped dividing, while those that did not were killed by the ampicillin. The cells were washed and resuspended in minimal medium. There was a significant reduction of over 80% in CFU formation for cultures grown in the presence of the *folA* siHybrids. There was an insignificant difference between the amount of CFUs formed in the positive control, cultures grown in the presence of the *folA* siRNA, and cultures grown in the presence of the non-homologous siHybrid. Subsequent addition of nucleosides to the minimal medium greatly stimulated growth as expected, because of the decreased biosynthetic burden on the cells. The cultures grown in the presence of the *folA* siHybrids as well as the purines and pyrimidines (siHybrid/Rescue) exhibited over 100% CFU formation (**Figure 2**). We have experimentally determined (data not shown) that this is caused by the growth rates of the rescue cultures being significantly faster than that of the positive control. These experiments demonstrated that the effect was both specific and non-lethal, and not possible with conventional siRNA treatments.

To test directly whether siHybrid treatment reduces protein levels in a specific manner, *E. coli* MG1655 cultures were treated with either a siHybrid directed against the mammlian (G6PD) gene as a negative control, or a siHybrid directed against the *lacZ* gene encoding -galactosidase. Transcription from the *lac* promoter was induced with isopropyl -D thiogalactopyranoside. Immunoblotting with an anti -galactosidase antiserum shows that the *lacZ* siHybrid reduces -galactosidase to undetectable levels whereas the G6PD siHybrid treatment had no effect. The *lacZ* gene precedes *lacY* in the same operon, raising the question of whether targeting *lacY* might affect -galactosidase levels. When a *lacY* siHybrid is used, -galactosidase protein levels are decreased somewhat but not dramatically. We hypothesize that this is due to the fact that transcription and translation are coupled in prokaryotes, such that degradation of the *lacZ* mRNA may be protected by ribosome loading. Because bacterial mRNAs are polycistronic, further work will be necessary to delineate possible polar effects of siHybrid treatment in various

circumstances. We conclude that siHybrid treatment dramatically reduces protein levels in a specific manner in *E. coli*.

It should be noted that no transforming steps were used in these experiments; simply adding the siHybrid molecules to the medium was sufficient. We initially used electro-competent and chemically competent cells, and transformed them with the siHybrid molecules using standard techniques, but the results were unsatisfactory. Untransformed controls to which the siHybrids had been added, however, showed the effects described above. Subsequently, all results were obtained without any active transformation protocols. Anecdotally, we have ongoing experiments using many different types of bacteria. Some, which are known to be naturally competent (e.g. *salmonella XXX*), readily take up the siHybrids with no transformation steps. Others, which are known to have certain naturally competent phases of their life cycle (e.g. *bacillus subtilis* immediately post-germination and pre-sporulation) take up the siHybrids during those two phases, but require active transformation during other points of their growth. Yersenia pestis, which has not been shown to be naturally competent at any point, required transformation. In each of these types of bacteria, uptake of the siHybrids was demonstrated by functional assays, including Western blots and growth curves (data not shown, as the work is in progress in other groups). Interestingly, not all strains of demonstrated the ability to take up the siHybrids without transformation. We have no explanation as to why these particular strains can; no data exist to support any hypothesis. It is possible that the siHybrids are both small and stable enough, and delivered in high enough concentrations, that sufficient quantities are transported across the cell membrane to activate the RNA interference process. Adding either the single-stranded DNA or RNA components of the siHybrid molecule had no effect. Taken together, these results suggest that the effect was gene specific and required an RNA:DNA hybrid.

SiRNA molecules do not induce RNA interference in bacteria, and it is believed this is due to the presence of RNase III in the cells [1]. However, we have shown here that siHybrid molecules do induce a gene silencing effect in *E. coli*. This is despite the cellular presence of RNase H, which is known to degrade RNA:DNA hybrid molecules [reviewed in 7]. In order to help understand why this is the case, we studied commercially available *E. coli*. RNase H and RNase III nucleases (New England Biolabs, Beverly, MA) for their activity against these molecules. Equal amounts of siHybrid and siRNA targeted against the chloramphenicol acetyltransferase mRNA were incubated with 1 U E. coli nuclease, the results of which are shown in **Figure 4**. It is clear from these results that RNase H does not digest siHybrid molecules, while RNase III does readily digest the siRNA molecules to <20 bp in length. This length is shorter than what has been shown as effective in inducing RNA interference in mammalian cells [4, 12]. We repeated the RNase H digestion with up to 5x more nuclease, without digesting the siHybrid (data not shown). These digestions were then repeated with several other siHybrid and siRNA sequences, all with the same results. RNase H failed to digest any siHybrid molecule and RNase III always digested the siRNA (data not shown). These data strengthen the idea that the siHybrid molecules may possess an increased stability within the bacterial cells.

This study shows that short RNA:DNA hybrids mediate specific gene silencing in *E. coli*. The mechanism by which this occurs is unclear and is an important area for further study. The observation that siHybrids also effect gene silencing in mammalian cells [11] suggests that it is possible that there is an ancient, highly conserved mechanism is at work. It has been suggested that the siRNA effect in eukaryotes evolved as a defense mechanism against eukaryotic double-stranded viruses. If this is indeed a much more conserved mechanism than previously thought, and assuming the machinery for the siHybrid activity is the same as that for

siRNA, we propose that the mechanism more likely originally evolved as a defense mechanism against bacteriophages that have RNA:DNA intermediates as part of their replication mechanism. The extent to which the underlying processes are conserved in prokaryotes is another important question, particularly in light of the extensive degree of microbial diversity. The results reported here suggest that siHybrids will serve as an important research tool for prokaryotic biology, and suggest the exciting possibility of a new class of antibacterial agents for treating infectious diseases.

Figures

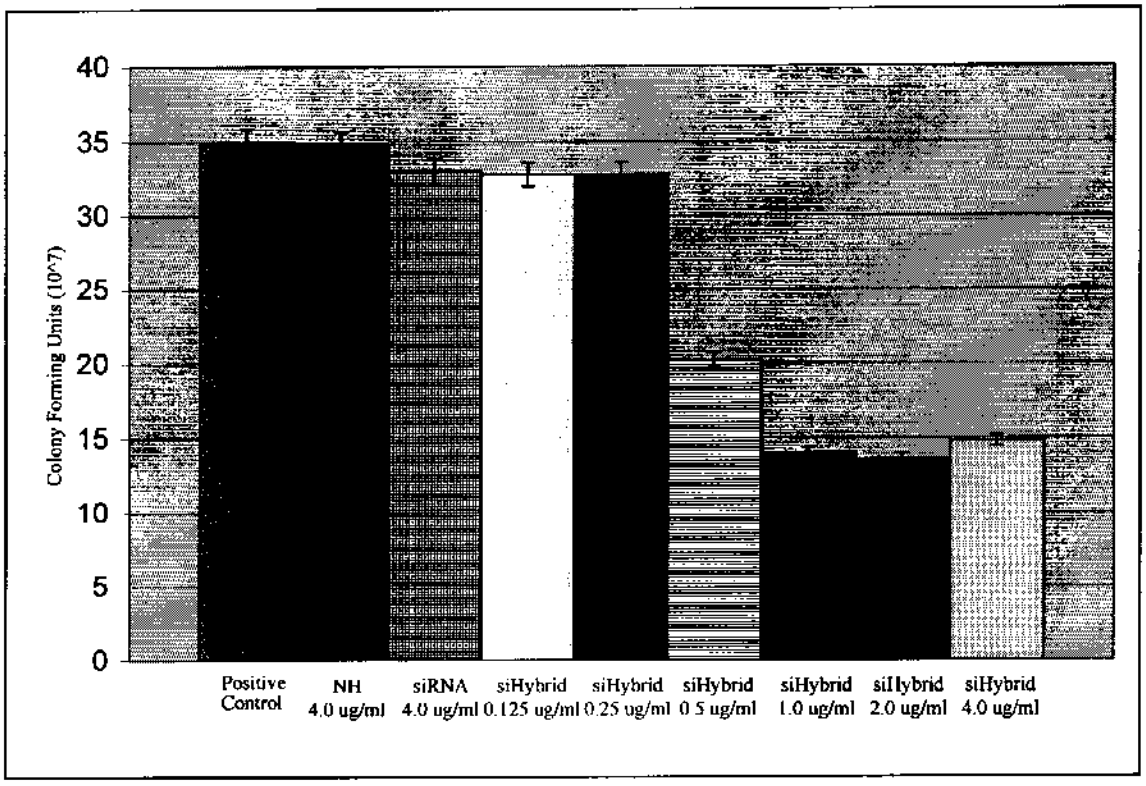

FIGURE 1 A: Sequence specific gene silencing of the Tn9 chloramphenicol acetyl transferase gene in E. coli transformed with the pBC SK+ plasmid encoding the CAT protein. *Varying doses of Tn9 cat siHybrid suppress gene function.*

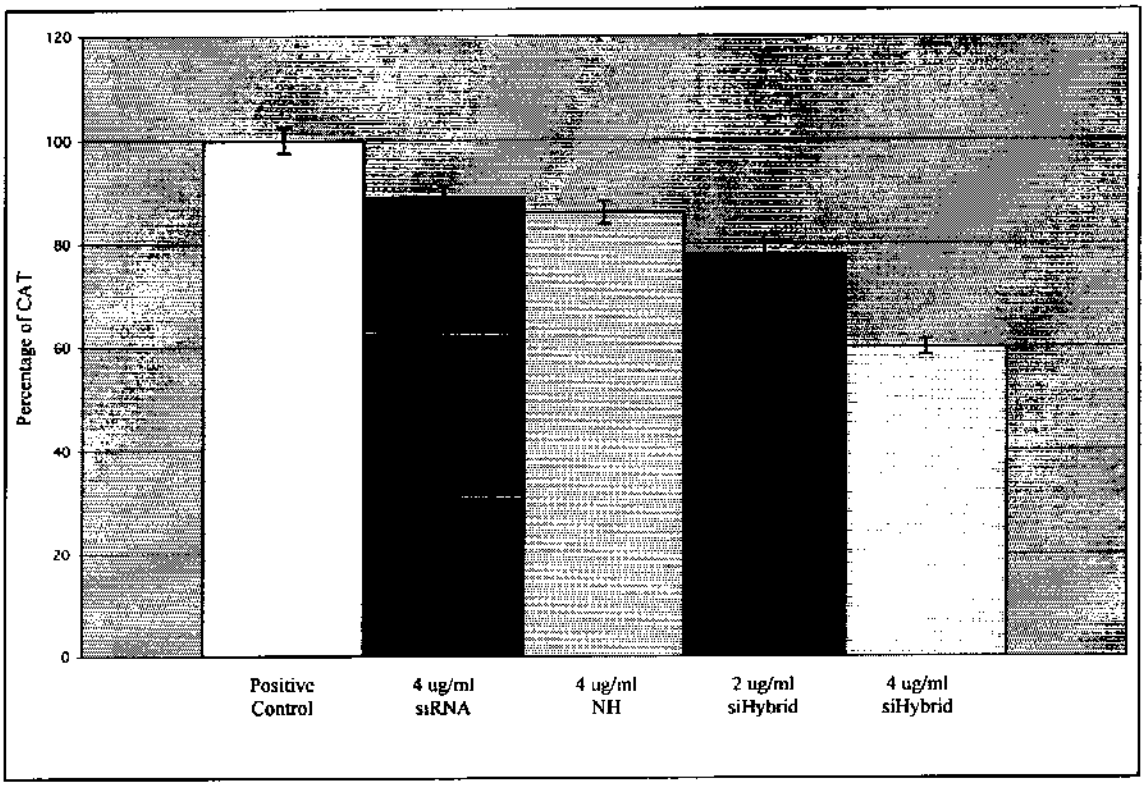

FIGURE 1: Sequence specific gene silencing of the Tn9 chloramphenicol acetyl transferase gene in E. coli transformed with the pBC SK+ plasmid encoding the CAT protein. *Tn9 cat siHybrids reduce the level of CAT protein produced in the cell.*

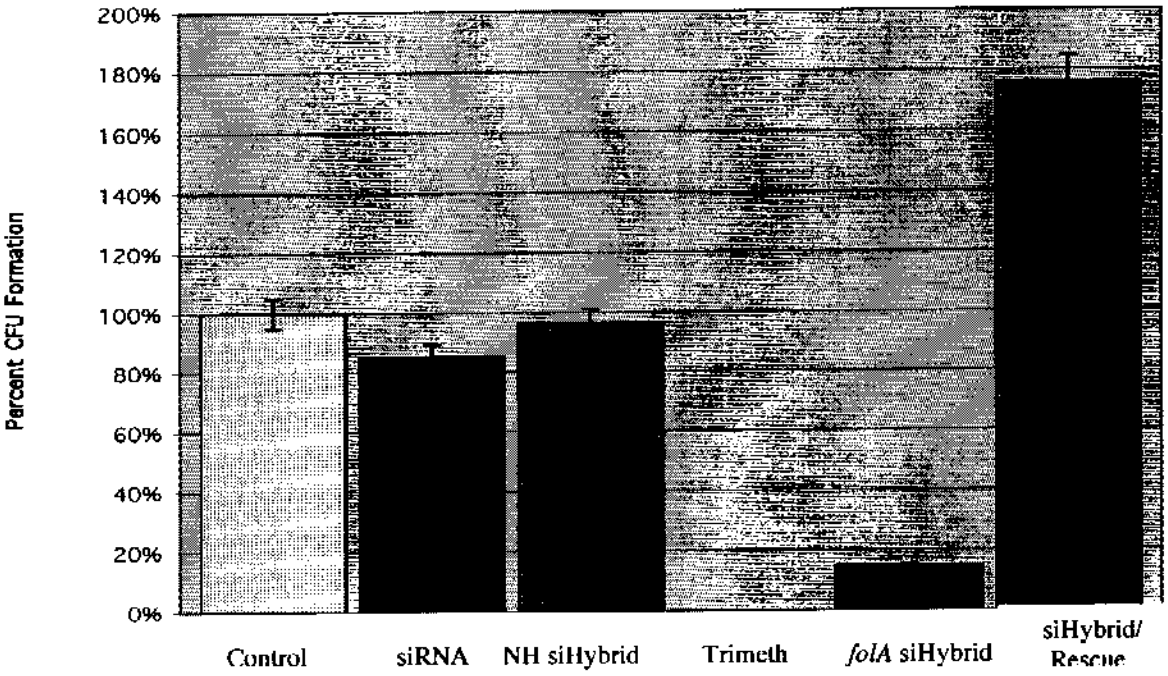

FIGURE 2: siHybrids suppress gene function without compromising the vitality of the cell.

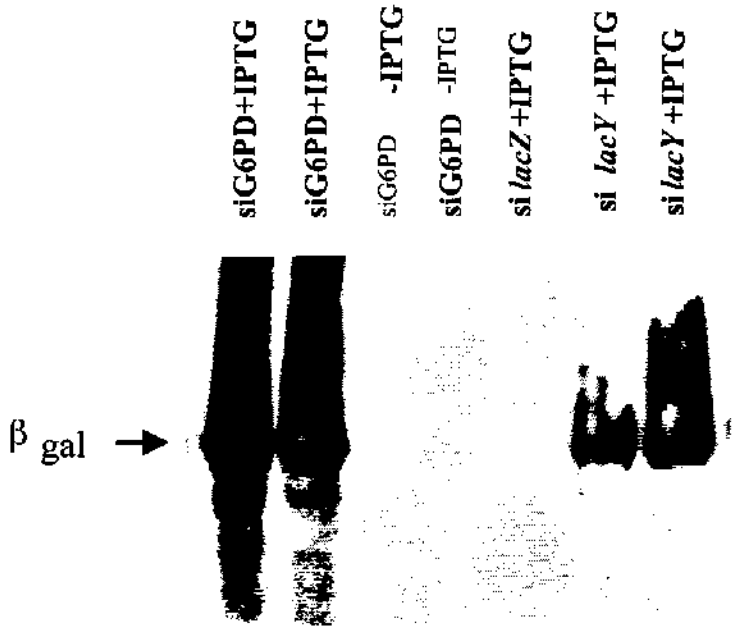

FIGURE 3: Reduction of β-galactosidase levels using a *lacZ* siHybrid

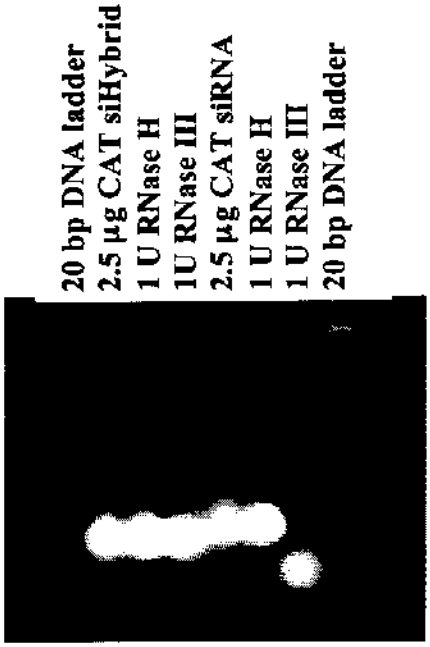

FIGURE 4. RNase H and III digestion of siHybrid and siRNA molecules.

Exit Plan

Our goal for the siHybrids is to develop them as a commercial gene silencing product for bacteria. We have demonstrated that they work in bacteria, but must demonstrate the mechanism thereof prior to commercialization. Specifically, we need to demonstrate that mRNA is cut by this method. We are working to do so as part of a different program, and hope for results shortly. Upon successful demonstration of the mRNA degradation, we will publish and, hopefully, license the technology.

Summary

Gene silencing using RNA interference (RNAi) is a new technique that has revolutionized laboratory research and clinical therapy. RNAi can turn off any gene, without any other effects. siHybrids, the novel technology developed at LLNL, offers a significantly increased silencing effect and duration compared to siRNA, the current technology. Further, siHybrids are effective in bacteria, and much more stable and cheaper to produce than siRNA. These key differences give siHybrids a distinct advantage over the current technology. The technology has a patent pending, and is the recipient of a 2004 R&D100 award.

Acknowledgements (if applicable)

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